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Irreversible Binding of Chlorinated Ethylenes to Macromolecules

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Rats have been exposed in a closed system to the chlorinated ethylenes vinyl chloride and trichloroethylene and to carbon tetrachloride as a reference compound. Data of uptake of the compounds, of urinary excretion of metabolites, and of exhalation after exposure show that the chlorinated ethylenes are metabolized much faster than carbon tetrachloride, probably due to their common ethylene structure. To eliminate differences in uptake, calculation of metabolites of the three compounds in tissues was based on the amount actually taken up by the animals. Vinyl chloride, trichloroethylene, and carbon tetrachloride showed irreversible binding of metabolites to tissue proteins, mainly of the liver. Irreversible protein binding of either of these compounds ranged within the same order of magnitude, if related to the amount of compound which had been taken up. Also, no differences in the relative portion of irreversibly bound metabolites were found after exposure to different atmospheric concentrations of the three compounds. As already shown for vinyl chloride, trichloroethylene is metabolized *in vitro* by rat liver microsomes in presence of NADPH-regenerating system to intermediates that irreversibly bind to proteins. Albumin (bovine and rabbit) was a preferred target for binding. In contrast to vinyl chloride, significant irreversible binding of trichloroethylene metabolites also occurred to non-SH-proteins (γ-globulin, concanavalin A) and to polylysine. Hence it should be inferred that, unlike vinyl chloride, trichloroethylene metabolites not only bind to sulfhydryl groups but also, to a lesser extent, to free amino groups of proteins.

Introduction

The carcinogenic properties of vinyl chloride (*1, 2*) and the possible (*3, 4*) carcinogenicity of trichloroethylene, along with the mutagenic effects of both compounds (*5*) has stimulated research on metabolism of the halogenated ethylenes. Accumulating evidence strongly suggests that both vinyl chloride and trichloroethylene are initially metabolized to their epoxide derivatives which undergo rearrangement to secondary metabolites or may react with nucleophilic components (*3, 5–7*). Interest has been focussed on covalent interaction of reactive metabolites of vinyl chloride and trichloroethylene with macromolecules, i.e., proteins (*8–11*) and nucleic acids (*8, 12*). Although covalent binding of metabolites of either of these compounds to tissue proteins is well established, comparative data, elaborated under identical experimental conditions, are lacking. This paper, therefore, compares the irreversible binding of metabolites of vinyl chloride and trichloroethylene to tissue proteins of the rat *in vivo*. As a reference compound, carbon tetrachloride has been included in the study. The data obtained *in vivo* are supported by those of *in vitro* experiments with rat liver microsomes.

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Materials and Methods

Materials

1,2-^{14}C-Vinyl chloride, specific radioactivity 10.7 mCi/nmole, was synthesized by the Radiochemical Department of Farbwerke Hoechst, Frankfurt, Germany. 1,2-^{14}C-Trichloroethylene (specific radioactivity 1.7 mCi/nmole) was purchased from New England Nuclear, Boston, Mass., and ^{14}C-carbon tetrachloride was purchased from the Radiochemical Centre, Amersham, England.

Exposure of Rats

Male Wistar rats, 200-250 g, were exposed in a closed all-glass system (volume 10.3 liters) to an atmosphere containing the ^{14}C-labeled halogenated hydrocarbon. This system has been described elsewhere (*13*). Decline of radiactivity in the atmosphere of the system was followed by liquid scintillation counting (*13*). Concentration of haloge-

nated hydrocarbon in the atmosphere was also followed by gas-liquid chromatography (GLC). Samples of 10 ml air were drawn from the system by means of syringe, and introduced into the 5 ml gas sampling loop of a six-port valve connected to a gas chromatograph (Varian 1400 with a flame ionization detector and a 3-m steel column, filled with Porapak Q). Retention times were 4.3 min for trichloroethylene at 230°C and 5.5 min for carbon tetrachloride at 200°C. Determination of radioactive metabolites in tissues was done as described previously (*13*). Irreversibly protein bound radioactive metabolites were measured after precipitation of proteins with ethanol and exhaustive extraction, as described elsewhere (*9*). Radioactivity in urine was determined by liquid scintillation counting in Bray's solution (*14*). To facilitate collection of the urine, the rats received five hourly doses of 5 ml tap water via a stomach tube (*15*).

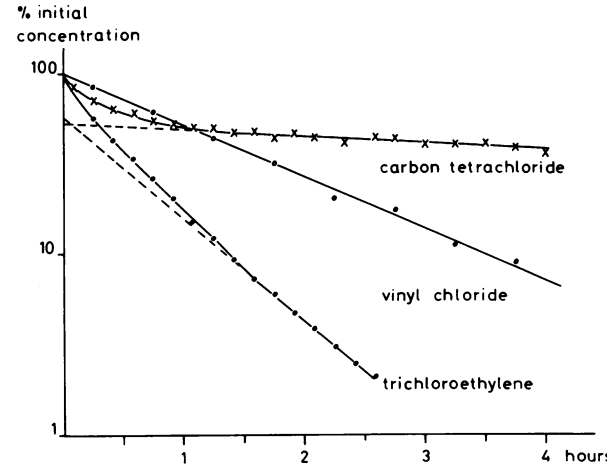

FIGURE 1. Decline of concentration of atmospheric vinyl chloride, trichloroethylene, and carbon tetrachloride in a closed system (volume (10.3 1) if occupied by three male Wistar rats; (--) extrapolated to zero time.

Incubations *in Vitro*

1,2-^{14}Trichloroethylene vapor was incubated in an all-glass incubation system with rat liver microsomes and NADPH-regenerating system as described elsewhere (*10, 11*). Soluble proteins were added to the microsomal incubation, and irreversible binding of trichloroethylene metabolites to these proteins was determined. After incubation the microsomes were separated from the soluble protein, precipitated with ethanol, and exhaustively extracted with organic solvents as described in detail (*16*) when labeled estrogens were used as substrate.

Results

Rates of Metabolism of Vinyl Chloride, Trichloroethylene and Carbon Tetrachloride *in Vivo*

Rats were exposed in a closed system to different initial concentrations of the halogenated olefins vinyl chloride and trichloroethylene and to the reference compound carbon tetrachloride. Decline of these compounds in the atmosphere of the system was determined by gas-liquid chromatography. Figure 1 demonstrates the typical decline curves, if the system (volume 10.3 1; 13) was occupied by three male Wistar rats (250 g), and if initial concentrations of the halogenated compounds were applied which do not saturate the metabolizing enzyme systems. Saturation of the metabolizing systems is achieved at 250 ppm vinyl chloride (*15*). The corresponding values for trichloroethylene and carbon tetrachloride are 150 ppm and 250 ppm, respectively. Whereas atmospheric vinyl chloride equilibrates with the animal organism within 15 min (*15*), equilibration of trichloroethylene and also of carbon tetrachloride takes much more time. Figure 1 shows that the linear slope of the decay curve of trichloroethylene is reached after 1.5 hr, and that the distributing phase of carbon tetrachloride lasts 1 hr. However, when the linear slopes of the curves, which are related to the rate of metabolism of the appropriate compound, are compared, it is apparent that the halogenated olefins vinyl chloride ($t_{1/2}$ = 1.1 hr) and trichloroethylene ($t_{1/2}$ = 0.55 hr) are metabolized at a much faster rate than carbon tetrachloride ($t_{1/2}$ = 7.3 hr). This is also reflected by the rate of urinary excretion of metabolites. When rats were exposed to ^{14}C-labeled vinyl chloride for 1 hr in our exposure system, 70% of the radioactivity was excreted *via* urine within 24 hr (*13, 15*). By contrast, only about 18% of ^{14}C-carbon tetrachloride which enters the organism via lung within 90 min is excreted by the kidneys during the first day after exposure (Fig. 2). The peculiar behavior of carbon tetrachloride, which apparently differs from that of the two chlorinated olefins, is also demonstrated in Figure 3. Three rats were exposed to 100 ppm ^{14}C-carbon tetrachloride vapor. The concentration of radioactivity in the system's atmosphere was determined throughout the entire experiment. After 1.5 hr when 50% of the radioactivity had disappeared from the gas phase and entered into the animal's organism (after this time the equilibration phase is ended, see Fig. 1), the exposure system was aerated for 1 min and all the

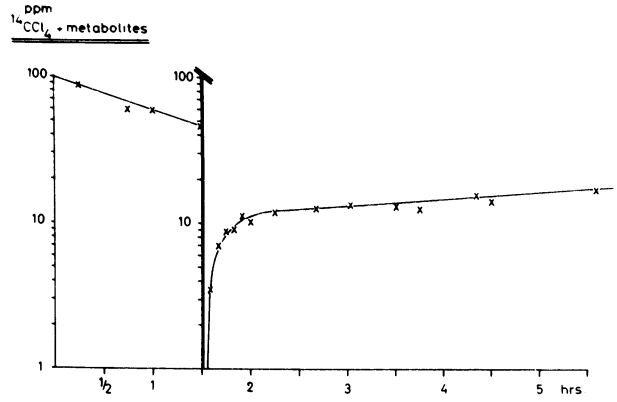

FIGURE 2. Excretion of radioactive metabolites in urine of rats after exposure to ^{14}C-carbon tetrachloride. Excretion is much less than after exposure to ^{14}C-vinyl chloride (*13*).

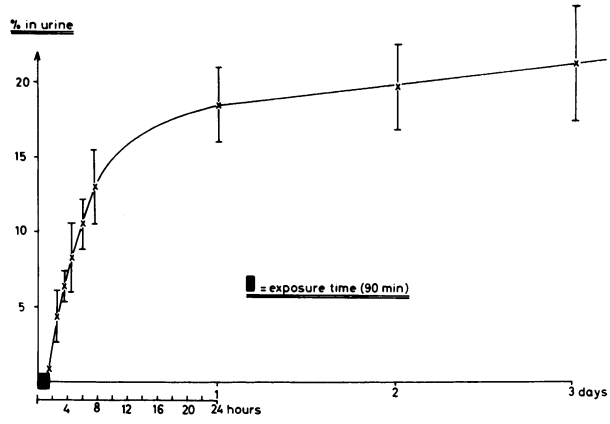

FIGURE 3. Radioactivity in the atmosphere of the closed exposure system on exposure of rats to ^{14}C-carbon tetrachloride. After 90 min, the system was aerated and exhalation of radioactivity was measured.

radioactivity present in the gas phase was removed. Then, expiration of radioactivity was followed over 4 hr. During this period, nearly 20% of the $^{14}CCl_4$ present initially, i.e., about 40% of that which was taken up by the organism, was exhaled. This also demonstrates that carbon tetrachloride, in contrast to the chlorinated ethylenes, is only slowly metabolized by rats, although it has considerable affinity for rat tissues, probably due to its lipophilicity. This may underline the importance of the ethylene structure of vinyl chloride and trichloroethylene for their metabolic fate in the organism.

Irreversible Binding to Tissue Proteins *in Vivo*

It is well established that metabolites of vinyl chloride (*8, 9, 11*) and trichloroethylene (*10, 17*) irreversibly (or covalently) bind to proteins. The same has also been reported for compounds like carbon tetrachloride (*18–21*) or halothane (*22–24*). It has been speculated that covalent binding of metabolites of a xenobiotic ought to be related to a possible carcinogenic potential of that compound (*25*). Therefore, covalent protein binding of metabolites of the halogenated olefins vinyl chloride and trichloroethylene and of carbon tetrachloride has been compared. All these compounds were administered to rats in gaseous (or vapor) form by exposing the animals in a closed system to different initial concentrations. Because of the different rates of uptake (see Fig. 1), it was necessary to base the calculation of metabolites in tissues on the amount of substrate which was actually taken up by the animals. Therefore, the data of total metabolites in tissues and of irreversibly protein bound metabolites were calculated as percent of that amount which was taken up. This procedure eliminated differences which could have been introduced by the different pharmacokinetics of the compounds. The results of the experiments are shown in Tables 1–3.

Radioactivity following exposure to ^{14}C-vinyl chloride is mainly concentrated in liver, the organ of metabolism, and in the kidneys, the organ of excretion of metabolites (Table 1). Furthermore, the small intestine contains substantial amounts of vinyl chloride-derived radioactivity. The main organ in which covalent protein binding takes place is the liver. Smaller amounts of irreversibly protein bound metabolites are found in kidneys, small intestine, in lung and in spleen. When the figures obtained after exposure to the different initial concentrations of vinyl chloride are compared, no significant differences in both the relative content of metabolites and of irreversibly protein bound metabolites are evident. Similar data as with vinyl chloride could also be obtained with ^{14}C-trichloroethylene (Table 2). In general, somewhat less total radioactivity is present in the tissues, but the covalent binding data are closely similar to those of vinyl chloride. For trichloroethylene, also, the main site of covalent protein binding is liver. As in vinyl chloride, no major differences are found, if different initial atmospheric concentrations of trichloroeethylene are applied.

Surprisingly, all the data for carbon tetrachloride (Table 3), which is not an ethylene derivative range within the same order of magnitude as those of vinyl chloride and trichloroethylene. For this compound also, the largest amount of radioactive metabolites, and of covalently protein bound metabolites is present in the liver.

Table 1. Total and irreversibly protein-bound metabolites of ^{14}C-vinyl chloride (VC) in different tissues of rats after 5 hr exposures.[a]

	% of radioactivity taken up/g tissue					
	VC = 2 ppm, n=4		VC=100 ppm, n=4		VC=1000 ppm, n=3	
Tissue	Total metabolites	Irreversibly bound	Total metabolites	Irreversibly bound	Total metabolites	Irreversibly bound
Lung	0.21 ± 0.033	0.08 ± 0.004	0.25 ± 0.012	0.08 ± 0.011	0.23 ± 0.015	0.07 ± 0.007
Liver	1.08 ± 0.13	0.32 ± 0.046	0.98 ± 0.105	0.32 ± 0.064	0.97 ± 0.166	0.37 ± 0.064
Spleen	0.24 ± 0.026	0.06 ± 0.005	0.31 ± 0.031	0.08 ± 0.023	0.20 ± 0.014	0.06 ± 0.008
Kidney	1.00 ± 0.17	0.12 ± 0.011	0.91 ± 0.13	0.11 ± 0.018	0.89 ± 0.097	0.09 ± 0.013
Small intestine	1.07 ± 0.092	0.10 ± 0.012	0.98 ± 0.18	0.09 ± 0.023	1.31 ± 0.50	0.10 ± 0.023
Muscle	0.13 ± 0.019	0.017 ± 0.002	0.17 ± 0.011	0.017 ± 0.010	0.17 ± 0.076	0.010 ± 0.076

[a]Male Wistar rats, 250 g; n = number of animals. Value shown are means ± SD.

Table 2. Total and irreversibly protein-bound metabolites of ^{14}C-trichloroethylene (TCE) in different tissues of rats after 5 hr exposure.[a]

	% of radioactivity taken up/g tissue					
	TCE = 9 ppm, n=4		TCE = 100 ppm, n=4		TCE = 1000 ppm, n=3	
Tissue	Total metabolites	Irreversibly bound	Total metabolites	Irreversibly bound	Total metabolites	Irreversibly bound
Lung	0.23 ± 0.026	0.06 ± 0.002	0.24 ± 0.025	0.06 ± 0.006	0.22 ± 0.055	0.1 ± 0.003
Liver	0.77 ± 0.059	0.28 ± 0.027	0.68 ± 0.073	0.27 ± 0.019	0.88 ± 0.046	0.48 ± 0.020
Spleen	0.14 ± 0.015	0.05 ± 0.002	0.15 ± 0.001	0.05 ± 0.004	0.15 ± 0.006	0.08 ± 0.003
Kidney	0.37 ± 0.005	0.09 ± 0.007	0.40 ± 0.029	0.09 ± 0.007	0.39 ± 0.045	0.14 ± 0.016
Small intestine	0.41 ± 0.058	0.05 ± 0.010	0.38 ± 0.062	0.07 ± 0.008	0.28 ± 0.015	0.09 ± 0.015
Muscle	0.11 ± 0.005	0.014 ± 0.001	0.11 ± 0.013	0.012 ± 0.001	0.10 ± 0.011	0.027 ± 0.003

[a]Male Wistar rats, 250 g; n=number of animals. Values shown are means ± SD.

Table 3: Total and irreversibly protein-bound metabolites of ^{14}C-carbon tetrachloride (CCl_4) in different tissues of rats after 5 hr exposure.[a]

	% of radioactivity taken up/g tissue			
	CCl_4 = 5 ppm, n=4		CCl_4 = 100 ppm, n=4	
Tissue	Total metabolites	Irreversibly bound	Total metabolites	Irreversibly bound
Lung	0.075 ± 0.018	0.033 ± 0.006	0.15 ± 0.017	0.036 ± 0.0051
Liver	0.81 ± 0.056	0.38 ± 0.01	1.17 ± 0.233	0.34 ± 0.060
Spleen	0.042 ± 0.011	0.016 ± 0.003	0.13 ± 0.004	0.024 ± 0.002
Kidney	0.29 ± 0.11	0.076 ± 0.004	0.63 ± 0.116	0.076 ± 0.012
Small intestine	0.22 ± 0.097	0.034 ± 0.003	0.46 ± 0.190	0.05 ± 0.0065

[a]Make Wistar rats, 250 g; n=number of animals. Values shown are means ± SD.

Irreversible Protein Binding *in Vitro*

It has been shown (*26*) that metabolites of vinyl chloride bind to free sulfhydryl groups of proteins. If ^{14}C-vinyl chloride is incubated with rat liver microsomes, NADPH-regenerating system and concanavalin A, a protein which contains no sulfur atoms at all, no irreversible binding occurs to this particular protein (*8*). However, some binding occurs to concanavalin A if ^{14}C-vinyl bromide is taken instead of vinyl chloride (unpublished data).

Table 4 shows the irreversible protein binding of ^{14}C-trichloroethylene metabolites formed *in vitro* from trichloroethylene by rat liver microsomes. Large amounts of metabolites bind to albumin (bovine and rabbit). Binding is reduced by glutathione, probably by reaction with reactive metabolites. Significant binding is also observed to non-SH proteins (μ-globulin, concanavalin A), although these bind less than albumin. These data, which differ from those obtained with vinyl chloride, are supported by a relatively high covalent binding capacity of the synthetic "protein" polylysine for trichloroethylene metabolites. This means that, in contrast to vinyl chloride (*8, 26*), tricholoroethylene metabolites are likely to irreversibly attach not only to SH groups, but also to a lesser extent, to NH_2 groups of protein.

Table 4. Irreversible binding of trichloroethylene metabolites to soluble proteins added to incubations of rat liver microsomes and NADPH-regenerating system.[a]

Protein	% of BSA binding
Bovine serum albumin (BSA)	100
BSA + 1 mM glutathione	39.7 ± 10.7
BSA without NADPH reg. system	20.3 ± 2.7
Rabbit serum albumin	105.0 ± 11.0
Bovine fibrinogen	39.8 ± 6.0
Bovine γ-globulin	35.8 ± 4.4
Human γ-globulin	50.0 ± 4.6
Concanavalin A	26.0 ± 2.0
Polylysine	42.8 ± 2.3

[a]Partial pressure of ^{14}C-trichloroethylene vapor in the gas phase was 1.11 mm Hg (saturation conditions). Binding is given as percentage of that observed for bovine serum albumin (BSA). Binding to 5 mg BSA was 0.28 ± 0.02 nmole/mg microsomal protein/hr. Values shown are means ± SD; n=4.

Discussion

The data show that vinyl chloride and trichloroethylene, if animals are exposed to these compounds, differ in their pharmacokinetic properties. Trichloroethylene needs more time than vinyl chloride to equilibrate with the organism, and is faster metabolized. However, the observed half-life (which is related to metabolic processes) of carbon tetrachloride is very much longer than that of the two chlorinated ethylenes examined. The more rapid metabolism of vinyl chloride and of trichloroethylene may be related to the common primary metabolic step which both compounds are thought to share, i.e., the formation of the epoxide intermediate (*3, 5–12, 27*). These epoxides also are probably involved in covalent binding to nucleophilic centers of macromolecules (*3, 8, 12, 27*). The present data show that quantitatively, with respect to covalent protein binding *in vivo*, vinyl chloride, trichloroethylene and also carbon tetrachloride behave similarly. All these compounds, which are metabolically activated in liver, show greatest covalent protein binding of metabolites in this particular organ. As with the carcinogenicity of vinyl chloride (*1, 2*) and of carbon tetrachloride (*21*), the present data for trichloroethylene suggest strongly that the possible carcinogenic effects of trichloroethylene (*4*) be considered seriously.

Covalent protein binding of metabolites of a xenobiotic, however, appears only to be a tool that allows us to detect whether reactive, and possibly hazardous, metabolites are formed. In addition, interaction with nucleic acids moieties has to be examined. For vinyl chloride, it could be demonstrated that adenine moieties are transformed to 1-N^6-ethenoadenine (*12*) and cytosine moieties to 3-N^4-ethenocytosine (unpublished data). For carbon tetrachloride, covalent binding to nucleic acids has also been reported (*21*). Furthermore, covalent binding to cytosolic pyridine nucleotides occurred (*28*). Hence, further studies are needed to show whether also trichloroethylene metabolites may interact with nucleotide bases and/or nucleic acids.

REFERENCES

1. Maltoni, C., and Lefemine, G. Carcinogenicity bioassays of vinyl chloride. Environ. Res. 7: 387 (1974).
2. Maltoni, C., and Lefemine, G. Carcinogenicity bioassays of vinyl chloride: current results. Ann. N. Y. Acad. Sci. 246: 195 (1975).
3. Van Duuren, B. L. On the possible mechanism of carcinogenic action of vinyl chloride. Ann. N. Y. Acad. Sci. 246: 258 (1975).
4. National Cancer Institute, U. S. DHEW Carcinogenesis bioassay of trichloroethylene. Carcinogenesis Technical Report Series No. 2. Government Printing Office, Washington, D. C., 1976.
5. Greim, H., et al. Mutagenicity *in vitro* and potential carcinogenicity of chlorinated ethylenes as a function of metabolic oxirane formation. Biochem. Pharmacol. 24: 2013 (1975).
6. Uehleke, H., et al. Spectral evidence for 2,2,3-trichlorooxirane formation during microsomal trichloroethylene oxidation. Xenobiotica 7: 94 (1977).
7. Jaeger, R. J., et al. Acute hepatic injury by vinyl chloride in rats pretreated with phenobarbital. Nature 252: 724 (1974).

8. Kappus, H., et al. Rat liver microsomes catalyse covalent binding of ^{14}C-vinyl chloride to macromolecules. Nature 257, 134 (1975).
9. Bolt, H. M., et al. Metabolism of ^{14}C-vinyl chloride *in vitro* and *in vivo*. Paper presented at symposium on environmental pollution and cancerogenic risks, Lyon, 1975; INSERM Symposia Series 52: 151 (1976).
10. Bolt, H. M., et al. Incubation of ^{14}C-trichloroethylene vapor with rat liver microsomes: uptake of radioactivity and covalent protein binding of metabolites. Int. Arch. Occup. Environ. Health 39: 103 (1977).
11. Kappus, H., et al. Liver microsomal uptake and transformation to protein alkylating metabolites *in vitro*. Toxicol. Appl. Pharmacol. 37: 461 (1976).
12. Laib, R. J., and Bolt, H. M. Alkylation of RNA by vinyl chloride metabolites *in vitro* and *in vivo:* Formation of 1-N^6-ethenoadenosine. Toxicology, in press.
13. Bolt, H. M., et al. Disposition of 1,2-^{14}C-vinyl chloride in the rat. Arch. Toxicol. 35: 153 (1976).
14. Bray, G. A. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1: 279 (1960).
15. Bolt, H. M., et al. Pharmacokinetics of vinyl chloride in the rat. Toxicology 7: 179 (1977).
16. Kappus, H., Bolt, H. M., and Remmer, H. Irreversible protein binding of metabolites of ethynylestradiol *in vivo* and *in vitro*. Steroids 22: 203 (1973).
17. Van Duuren, B. L., and Banerjee, S. Covalent interaction of metabolites of the carcinogen trichloroethylene in rat hepatic microsomes. Cancer Res. 36: 2419 (1976).
18. Uehleke, H., and Werner, T. A comparative study on the irreversible binding of labelled halothane, trichlorofluoromethane, chloroform, and carbon tetrachloride to hepatic protein and lipids *in vitro* and *in vivo*. Arch. Toxicol. 34: 289 (1975).
19. Uehleke, H., Hellmer, K. H., and Tabarelli, S. Binding of ^{14}C-carbon tetrachloride to microsomal proteins *in vitro* and formation of $CHCl_3$ by reduced liver microsomes. Xenobiotica 3: 1 (1973).
20. Diaz Gomez, M. I., et al. Irreversible binding of ^{14}C from $^{14}CCl_4$ to liver microsomal lipids and proteins from rats pretreated with compounds altering microsomal mixed function oxygenase activity. Toxicol. Appl. Pharmacol. 25: 534 (1973).
21. Rocchi, P., et al. *In vivo* and *in vitro* binding of carbon tetrachloride with nucleic acids and proteins in rat and mouse liver. Int. J. Cancer 11: 419 (1973).
22. Uehleke, H., Hellmer, K. H., and Tabarelli-Poplawski, S. Metabolic activation of halothane and its covalent binding to liver endoplasmic proteins *in vitro*. Naunyn-Schmiedeberg's Arch. Pharmacol. 279: 39 (1973).
23. Van Dyke, R. A., and Wood, C. L *In vitro* studies on irreversible binding of halothane metabolite to proteins. Drug Metab. Dispos. 3: 51 (1975).
24. Hempel, V., and Remmer, H. *In vivo* and *in vitro* studies on irreversible binding of halothane metabolites to proteins. Experientia 31: 680 (1975).
25. Miller, J. A. Cancerogenesis by chemicals. Cancer Res. 30: 559 (1970).
26. Bolt, H. M., et al. Untersuchungen zum Stoffwechsel von Vinylchlorid unter dem Blickpunkt der chemischen Karzinogenese. Verhandl. Deut. Ges. Arbeitsmed. 16: 181 (1976).
27. Bonse, G., et al. Chemical reactivity, metabolic oxirane formation, and biological reactivity of chlorinated ethylenes in the isolated perfused rat liver preparation. Biochem. Pharmacol. 24: 1829 (1975).
28. Harders, I., et al. Irreversible binding of $^{14}CCl_4$-metabolites to reduced phosphopyridine nucleotides *in vivo* and *in vitro*. Naunyn-Schmiedeberg's Arch. Pharmacol. (Suppl.) 293: R65 (1976).